INFECTION AND IMMUNITY, Mar. 1998, p. 1070–1075 Vol. 66, No. 3
0019-9567/98/$04.00+0

Transcriptional Regulation of Endothelial Cell Tissue Factor Expression during *Rickettsia rickettsii* Infection: Involvement of the Transcription Factor NF-κB

RUI-JIN SHI,[1] PATRICIA J. SIMPSON-HAIDARIS,[1] NORMA B. LERNER,[1] VICTOR J. MARDER,[1] DAVID J. SILVERMAN,[2] AND LEE ANN SPORN[1]*

Vascular Medicine Unit, Department of Medicine, Department of Pathology and Laboratory Medicine, Department of Microbiology and Immunology, and Department of Pediatrics, University of Rochester School of Medicine & Dentistry, Rochester, New York,[1] and Department of Microbiology and Immunology, University of Maryland School of Medicine, Baltimore, Maryland[2]

Received 2 September 1997/Returned for modification 21 October 1997/Accepted 8 December 1997

The vascular endothelial cell (EC) is a primary target of infection with *Rickettsia rickettsii*, the etiologic agent of Rocky Mountain spotted fever. Changes in gene transcription elicited by intracellular infection, including EC expression of the coagulation pathway initiator known as tissue factor (TF), may contribute to the vascular pathology observed during disease. Nuclear run-on analysis of uninfected and infected, cultured human endothelial cells revealed that the rate of TF mRNA transcription is enhanced more than twofold at 3 h following infection, thus coinciding with increased steady-state levels of TF mRNA. TF mRNA remained relatively unstable during infection, with a half-life of 1.6 h. The eukaryotic protein synthesis inhibitor cycloheximide did not block *R. rickettsii*-induced increase in TF mRNA levels and actually resulted in its superinduction, thus revealing that de novo synthesis of host cell protein was not prerequisite to this transcriptional response. Involvement of the transcription factor NF-κB in *R. rickettsii*-induced TF expression was demonstrated by using two unrelated inhibitors of NF-κB activation. The antioxidant pyrrolidinedithiocarbamate and the proteasome inhibitor *N*-tosyl-L-phenylalanine chloromethyl ketone blocked expression of TF mRNA and activity during infection. This study demonstrates that *R. rickettsii* infection results in transcriptional activation of the TF gene and that this response involves activation of the transcription factor NF-κB.

The vascular endothelial cell (EC) is a primary target of *Rickettsia rickettsii* infection during Rocky Mountain spotted fever, and the effects elicited by intracellular infection of this cell type likely contribute to the vascular pathology which is a hallmark of disease and which includes formation of fibrin microthrombi, enhanced vessel permeability, and vasculitis (38). Considerable experimental evidence exists to support the notion that in addition to necrotic cell injury, the *R. rickettsii*-infected EC alters its production of several proteins which likely results in presentation of a procoagulant and proinflammatory phenotype. Evidence for such responses is provided by studies of cultured EC, in which rickettsial infection cause increased expression of tissue factor (TF) (33, 36), plasminogen activator inhibitor 1 (9, 27), E-selectin (31), interleukin-1 (IL-1) (14, 36), and IL-6 and -8 (14).

TF, a 263-residue membrane glycoprotein, plays a critical role in initiation of blood coagulation by serving as an essential cofactor for activation of factor VII (2). TF is not normally expressed by cells of the vasculature but rather is present in many other tissues, thus forming a hemostatic envelope to induce coagulation in the event of vessel disruption (19). TF can be induced in cultured EC and in monocytes (25), and such expression by these vascular cells during disease likely results in thrombosis. Since activation of coagulation and resultant thrombus formation is a common occurrence in the course of Rocky Mountain spotted fever and may be a central pathophysiologic occurrence, much effort in our laboratory is directed toward gaining further understanding of the dynamics and mechanisms involved in *R. rickettsii*-induced TF expression.

Except for an apparent requirement for intracellular infection (33), the mechanisms governing *R. rickettsii*-induced TF expression, as well as expression of other rickettsia-induced proteins, have remained elusive. Endothelial TF expression, which is induced in cultured EC in response to soluble agonists such as cytokines (5, 6, 18), lipopolysaccharides (LPSs) (7), and phorbol esters (16), is controlled by transcriptional and post-transcriptional control mechanisms (1, 8, 10, 26). Increases in steady-state levels of TF mRNA in response to these soluble stimuli can occur in the absence of de novo protein synthesis (8), and thus TF is often described as an early-response gene. In this report, we analyze both the transcription rate and stability of TF mRNA during *R. rickettsii* infection and explore the requirement for de novo host cell protein synthesis. Further, we document involvement of the transcription factor NF-κB in *R. rickettsii*-induced TF expression. These studies provide the first direct evidence for *R. rickettsii*-induced activation of transcription of an EC gene and demonstrate that TF behaves as an early-response gene to this novel cell stimulus.

MATERIALS AND METHODS

EC culture, infection, and drug treatment. Human umbilical vein EC were cultured as previously described (11, 38), using umbilical cords collected within 48 h of delivery. Cells were cultured in McCoy's 5a medium (Flow Laboratories, McLean, Va.) containing 20% fetal bovine serum, EC mitogen (50 μg/ml; Collaborative Research, Inc., Bedford, Mass.), heparin (100 μg/ml), and insulin (25 μg/ml) (Sigma Chemical Co., St. Louis, Mo.). Cells at second passage were used in experimental protocols and were plated so as to achieve 80 to 90% confluency after 5 to 7 days in culture. *R. rickettsii* (Sheila Smith strain) was used as a plaque-purified seed stock (1×10^7 to 5×10^7 PFU/cm^2) prepared in Vero cells (African green monkey kidney; American Type Culture Collection, Rockville,

* Corresponding author. Mailing address: Vascular Medicine Unit, P.O. Box 610, University of Rochester Medical Center, 601 Elmwood Ave., Rochester, NY 14642. Phone: (716) 275-0439. Fax: (716) 473-4314. E-mail: Lee_Ann_Sporn@medicine.rochester.edu.

Md.) (29). EC were infected by using approximately 6×10^4 PFU/cm^2 of cell culture area. Infection was monitored by using EC plated on Thermanox coverslips (Ted Pella Inc., Tustin, Calif.) and stained by immunofluorescence using antibody against *R. rickettsii* (kindly provided by T. Tzianobos, Centers for Disease Control, Atlanta, Ga.) as previously described (32). EC cultures were preincubated with pyrrolidinedithiocarbamate (PDTC; 25 μM; Sigma) and *N*-tosyl-L-phenylalanine chloromethyl ketone (TPCK; 50 μM; Sigma) for 1 h prior to and during infection.

cDNA probes. The TF probe was prepared by using the *Eco*RI-*Hin*dIII fragment corresponding to bases 1 to 1358 subcloned from pKS2b (a kind gift from W. H. Konisberg, Yale University, New Haven, Conn.) into pGEM7Zf(−) (Promega, Madison, Wis.). The human γ-actin probe was obtained from L. Kedes (Stanford University, Palo Alto, Calif.). The 2,200-bp *Bam*HI fragment of γ-actin was subcloned into the *Bam*HI site of pGEM7Zf(−).

Northern blot analysis. EC cultured in 75-cm^2 flasks were infected for 4 h and then lysed with 6.4 ml of Tri-Reagent (Molecular Research Center, Inc., Cincinnati, Ohio), and RNA was isolated according to the manufacturer's instructions and dissolved in distilled water. Twenty micrograms of total RNA per condition was denatured with glyoxal-dimethyl sulfoxide and then resolved by electrophoresis in 1.2% agarose gels in 10 mM sodium phosphate buffer (pH 7.0) (30). Total RNA was prepared for Northern blot analysis by electrophoretic transfer to Zeta-Probe membranes (Bio-Rad, Centreville, N.Y.). Probes were labeled with [α-^{32}P]dCTP, using a random primer labeling kit from Life Technologies (Gaithersburg, Md.). To determine steady-state levels of TF or γ-actin, hybridization was carried out at 65°C with 0.5 M Na_2HPO_4-H_3PO_4 (pH 7.2)–1 mM EDTA–7% (wt/vol) sodium dodecyl sulfate (SDS), with final stringency washes carried out as described previously (12). For determination of mRNA stability, EC were infected with *R. rickettsii* for times indicated, then actinomycin D (20 μg/ml; Sigma) was added, and cultures were incubated at 37°C for additional times prior to RNA extraction and Northern blot analysis. Northern blotting was performed under conditions of probe excess, and several autoradiographic exposures were prepared to ensure that signals were below saturation. The amount of 18S rRNA was determined by scanning of a photographic negative prepared following acridine orange staining. For studies of the requirement for de novo protein synthesis in TF induction, EC were incubated with cycloheximide (CHX; 10 μg/ml; Sigma) for 1 h prior to and during infection. Total RNA was extracted and analyzed as described above.

Nuclear run-on assay. In vitro measurement of mRNA transcription rate was carried out by using a modification of previously described methods (8). Nuclei from approximately 4×10^7 cells/sample were isolated from second- or third-passage EC by the following method. EC were harvested by brief exposure to trypsin-EDTA (Gibco Life Technologies, Grand Island, N.Y.), collected into 15-ml polypropylene tubes, centrifuged at 500 × *g* for 5 min, and then resuspended in 10 ml of ice-cold resuspension buffer (RSB; 10 mM Tris-HCl [pH 7.4], 10 mM NaCl, 5 mM $MgCl_2$, 1 mM phenylmethylsulfonyl fluoride, 5 mM benzamidine). Cells were centrifuged and resuspended twice in RSB and then vortexed with gradual addition of 7 ml of lysis buffer (10 mM Tris-HCl [pH 7.4], 10 mM NaCl, 5 mM $MgCl_2$, 0.5% Nonidet P-40). Nuclei were pelleted at 500 × *g* for 5 min, washed twice with RSB, resuspended in 210 μl of storage buffer (50 mM Tris-HCl [pH 8.0], 40% glycerol, 5 mM $MgCl_2$, 0.1 mM EDTA), and stored at −70°C. In vitro transcription was carried out by mixing 200 μl of nuclei with 200 μl of 2× reaction buffer (10 mM Tris-HCl [pH 8.0], 5 mM $MgCl_2$, 4 mM $MnCl_2$, 0.3 M KCl, 10 mM ATP, 10 mM GTP, 10 mM CTP) and 250 μCi of [α-^{32}P]UTP (3,000 Ci/mmol; New England Nuclear, Boston, Mass.) for 30 min with shaking at 30°C. A volume of 600 μl of HSB buffer (10 mM Tris-HCl [pH 7.4], 0.5 M NaCl, 50 mM $MgCl_2$, 2 mM $CaCl_2$) containing 60 U of RQ1 DNase 1 (Promega) and 42 U of rRNasin (Promega) was added, after which the mixture was sheared by repeated pipetting and then incubated at 37°C for 20 min with shaking. Finally, 200 μl of Tris-SDS buffer (0.5 M Tris-HCl [pH 7.4], 125 mM EDTA, 5% SDS) containing 500 μg of proteinase K (Gibco Life Technologies) was added to each reaction mixture and incubated at 37°C for 45 min with shaking. Labeled RNA was isolated by phenol-chloroform extraction and ethanol precipitation and resuspended in 100 μl of H_2O containing 100 μl of S256 [100 μg of yeast RNA, 4 μg of poly(A), 4 μg of poly(C), and 25 μg of salmon sperm DNA in 100 ml of 33 mM Tris-HCl buffer (pH 8.0)]. Samples were boiled for 5 min to denature and quenched on ice. Target cDNAs were denatured in 0.4 M NaOH–10 mM EDTA, boiled for 10 min, and then quenched on ice, and an equal volume of ice-cold 2 M ammonium acetate (pH 7.0) was added to neutralize. The target cDNA (200 μl) was applied to Zeta-Probe membranes by using a slot blotter (Biodot SF; Bio-Rad Laboratories, Hercules, Calif.), UV cross-linked, and prehybridized with 0.5 M Na_2HPO_4-H_3PO_4 (pH 7.2)–1 mM EDTA–7% SDS for 1 h at 65°C with shaking. Membranes were hybridized with an equivalent amount of denatured, labeled RNA (1×10^6 to 5×10^6 cpm/ml) at 65°C for 72 h, then washed as described for Northern blot analysis, and exposed to X-Omat AR film (Eastman Kodak, Rochester, N.Y.) at −70°C.

Isolation of nuclei extraction, and gel shift assay. Following infection with *R. rickettsii*, nuclei were isolated and nuclear proteins were extracted as previously described (13, 15), using approximately 5×10^6 EC per experimental condition. The protein concentration in the nuclear extract was measured by using Bradford reagent. This procedure typically yielded protein concentrations between 1 and 2 mg/ml. HeLa cell nuclear extracts used as controls were obtained from Promega. Gel shift assays were performed by using the Promega gel shift assay system as instructed by the manufacturer, using 1 to 2 μg of protein obtained from the nuclear extractions for each gel shift reaction. Sequences of double-stranded consensus oligonucleotides used in the gel shift reactions were as follows: NF-κB (Promega), 5′-AGT TGA GGG GAC TTT CCC AGG C-3′; and AP-1 (Promega), 5′-CGC TTG ATG AGT CAG CCG GAA-3′. Probe labeling was carried out according to the manufacturer's instructions, using [γ-^{32}P]ATP (3,000 Ci/mmol; 10 mCi/ml; DuPont NEN Research Products, Boston, Mass.). Competition studies were performed with a 10-fold molar excess of unlabeled oligonucleotides added to reaction mixtures prior to addition of radiolabeled oligonucleotides. Reaction mixtures were analyzed on 4% nondenaturing, polyacrylamide gels prepared with 0.5× TBE (89 mM Tris-HCl [pH 8.0], 89 mM boric acid, 2 mM EDTA). The running buffer was 0.5× TBE. Gels were electrophoresed at 100 V for 3 h, and autoradiographic exposure of gels was for 12 to 18 h.

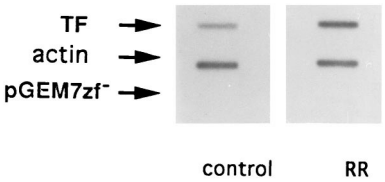

FIG. 1. Nuclear run-on assay for TF mRNA transcription rate in control and *R. rickettsii* (RR)-infected EC. In vitro transcription was carried out on nuclei isolated from 3-h-infected EC, using ^{32}P-labeled UTP, and then radiolabeled RNA was hybridized with immobilized target cDNA (TF, actin, or the pGEM-7Zf$^-$ cloning vector lacking an insert). No detectable hybridization occurred with the pGEM-7Zf$^-$ cloning vector alone.

Semiquantitative RT-PCR. Total cellular RNA was isolated from EC cultured in 25-cm^2 flasks, using Tri-Reagent (Molecular Research Center) as instructed by the manufacturer. Total RNA (0.5 μg) was reverse transcribed by using Superscript RNase H reverse transcriptase (10 U; BRL, Gaithersburg, Md.) with oligo(dT)$_{16}$ (2.5 μM) in a 20-μl reaction of 5 mM $MgCl_2$, PCR Buffer II (Perkin-Elmer, Madison, Wis.), 1 mM each of all four deoxynucleoside triphosphates, and RNase inhibitor (1 U) (Perkin-Elmer) and amplified by using a Gene Amp PCR System 9600 (Perkin-Elmer). Cycles consisted of an initial incubation at 95°C for 105 s and then cycling at 95°C for 30 s, 65°C for 30 s, and 72°C for 60 s, with a final incubation at 72°C for 7 min. For detection of IκBα mRNA, 15 μl of the reverse transcription reaction (RT) was amplified in a 100-μl reaction mixture. The primers used were as follows: IκBα forward primer, 5′-GCT CGG AGC CCT GGA AGC-3′; IκBα reverse primer, 5′-GCC CTG GTA GGT AAC TCT-3′ (566-bp product); TF forward primer, 5′-ACT CCC CAG AGT TCA CAC CTT ACC-3′; and TF reverse primer, 5′-TGA CCA CAA ATG CCA CAG CTC C-3′ (398-bp product). To normalize among samples, 2 μl of RT was amplified in a 100-μl reaction mixture, using primers for the housekeeping species glyceraldehyde-3-phosphate dehydrogenase (GAPDH): forward primer, 5′-CCA CCC ATG GCA AAT TCC ATG GCA-3′; and reverse primer, 5′-TCT AGA CGG CAG GTC AGG TCC ACC-3′ (588-bp product). To ensure that the PCR amplification had not reached plateau phase, the amplification products were compared following completion of 25, 30, and 35 cycles. Aliquots of PCR products were compared with a 1-kb DNA ladder (BRL), separated by electrophoresis on a 1.5% agarose gel, and visualized by ethidium bromide staining.

Assay of TF activity. EC cultured in 12-well culture plates were washed twice with Tris-buffered saline (0.05 M Tris, 0.1 M NaCl [pH 7.5]) and lysed in 0.16 ml of Tris-buffered saline with 10 mg of bovine serum albumin per ml. After repeated freeze-thawing, TF activity in the lysed cell sample was assayed using a two-stage clotting assay as previously described (32), and results were determined based on a standard curve generated by using pure human brain TF reconstituted into phospholipid vesicles as previously described (3, 23).

RESULTS

Nuclear run-on experiments were conducted to determine if *R. rickettsii* infection of EC increased the rate of transcription of the TF gene. Nuclei were extracted from uninfected and infected EC, in vitro transcription was conducted with ^{32}P-labeled UTP, and then radiolabeled products were hybridized with TF and γ-actin cDNA probes and with plasmid vector alone. The intensity of labeled actin mRNA in infected and uninfected EC was equivalent, yet intensity of labeled TF mRNA was increased (2.5-fold higher than the control value in this representative experiment), indicating an enhanced rate of transcription of the TF gene (Fig. 1). This observation is consistent with the increased steady-state level of TF mRNA observed at 4 h following *R. rickettsii* infection (32). The tran-

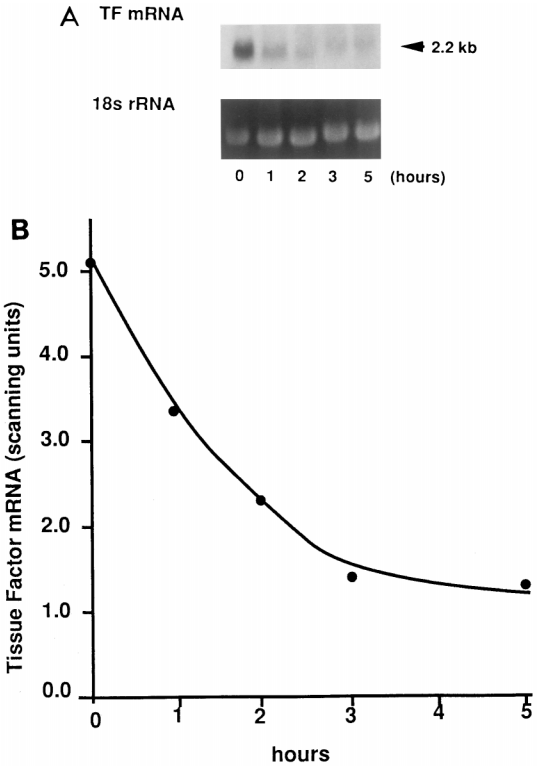

FIG. 2. Stability of TF mRNA in *R. rickettsii*-infected and uninfected EC. Actinomycin D (10 μg/ml) was added to EC 4 h after infection to inhibit further transcription, and then TF mRNA (2.2 kb) was analyzed by Northern blotting following incubation times of 0, 1, 2, 3, and 5 h (A). TF mRNA remaining at these time points was determined by densitometric scanning, the results of which were normalized to amounts of 18S rRNA contained in each sample and then expressed graphically in arbitrary units of optical density (B).

scription inhibitor actinomycin D (10 μg/ml), when present during infection, completely blocked *R. rickettsii*-induced expression of TF activity as measured by a two-stage clotting assay (not shown), providing further evidence that a transcriptional event even is necessary for *R. rickettsii*-induced expression of TF.

The half-life of TF mRNA in infected EC was measured to determine if mRNA stability was prolonged at the times of peak increases in steady-state mRNA levels. EC were infected for 3 h, and then actinomycin D (10 μg/ml) was added to inhibit further transcription. Total cellular RNA was harvested following further incubation for various time intervals and subjected to Northern blot analysis (Fig. 2A). TF mRNA content was quantitated by densitometric scanning and normalized to the amount of 18S rRNA. Scanning data, shown plotted on a linear scale in Fig. 2B, were subsequently linearized on semilog plots and subjected to regression analysis to calculate half-life values. TF mRNA in infected EC (0 h) was highly unstable, with a calculated half-life of approximately 1.6 h, based on a regression line with an r value of -0.993. Stability of TF mRNA in uninfected EC could not be determined since this mRNA was present at undetectable levels. *R. rickettsii* infection did not alter that half-life of the housekeeping mRNA species γ-actin, which was determined to be 9.5 h (data not shown). Therefore, change in stability of mRNA species was not a general cellular phenomenon.

The requirement for de novo host cell protein synthesis in *R. rickettsii*-induced expression of TF mRNA was explored by

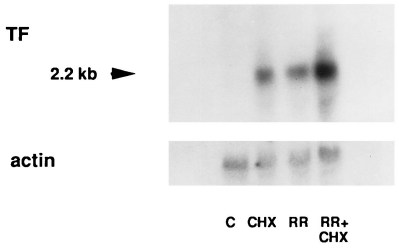

FIG. 3. Effect of CHX on EC expression of TF mRNA during *R. rickettsii* infection. The eukaryotic protein synthesis inhibitor CHX (10 μg/ml) was added to EC cultures during infection with *R. rickettsii* (RR) for 4 h, and then mRNA levels were determined by Northern blot analysis. Blots were then stripped and rehybridized with a cDNA probe against the housekeeping species human γ-actin. C, control.

using the eukaryotic protein synthesis inhibitor CHX (10 μg/ml) (Fig. 3). EC were infected in the presence and absence of CHX, treatment which does not inhibit entry of the organisms into EC (31). Consistent with the previously reported observation (8), Northern blot analysis of TF mRNA at 4 h demonstrated appearance of TF factor mRNA with CHX treatment alone (lane 2), whereas untreated EC contained nearly undetectable levels (lane 1). This effect of CHX treatment may derive from stabilization of this highly labile mRNA species or from enhanced transcription rate. *R. rickettsii* infection alone resulted in the appearance of TF mRNA (lane 3); however, superinduction occurred when CHX was present during infection (lane 4). This result indicated that induction of TF expression during infection occurred independently of de novo host cell protein synthesis, and thus host cell signalling involved in this response did not involve synthesis of a protein intermediate.

TF expression in cultured EC induced by various soluble agonists is dependent on activation of members of the NF-κB/Rel family of transcription factors (20–22), and it was recently reported that infection of cultured EC with *R. rickettsii* results in activation of NF-κB (33). To explore involvement of NF-κB activation in *R. rickettsii*-induced expression of TF, we used two inhibitors of NF-κB activation, the antioxidant PDTC (25 μM) (4, 20) and the proteasome inhibitor TPCK (50 μM) (17). Neither agent affected the initial rate of infection (Table 1) or resulted in any obvious changes in morphology of the infected cells (Fig. 4). Both agents were effective at blocking *R. rickettsii*-induced activation of NF-κB (Fig. 5A), which appears as two gel-shifted complexes representing a p50 homodimer (C1) and a p50-p65 heterodimer (C2) (24, 34), as determined by gel shift assay using a ^{32}P-labeled oligonucleotide probe corresponding to the κB binding domain of the murine kappa light-chain gene enhancer and as measured at 3 h following the

TABLE 1. EC infection with *R. rickettsii*[a]

Treatment	% EC infected	Avg no. of organisms/cell
R. rickettsii	87	3.9
R. rickettsii + PDTC (25 μM)	88	3.3
R. rickettsii + TPCK (50 μM)	81	3.8

[a] EC cultured on coverslips were infected in the absence or presence of inhibitors of NF-κB activation. At 6 h, EC were fixed and stained by fluorescence with anti-*R. rickettsii* antiserum. For each condition, 100 cells were selected randomly and scored for number of *R. rickettsii* organisms per cell.

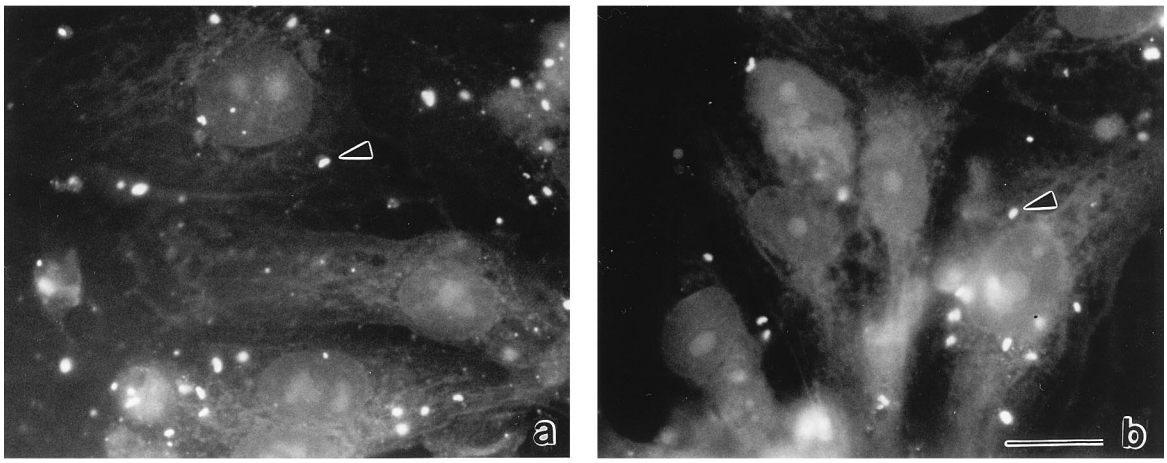

FIG. 4. Immunofluorescence staining of *R. rickettsii*-infected EC. EC cultured on plastic coverslips were infected for 6 h in the absence (a) or presence (b) of the proteasome inhibitor TPCK. Coverslips were then fixed and stained by fluorescence using polyclonal anti-*R. rickettsii* antibody. Arrowheads point to *R. rickettsii* organisms. Bar = 20 μm.

initiation of infection. These compounds specifically inhibited NF-κB activation, as there was no inhibitory effect on the level of activation of the unrelated transcription factor, AP-1 (not shown). TPCK (Fig. 5B) and PDTC (not shown), when present during infection, blocked *R. rickettsii*-induced NF-κB activation and prevented *R. rickettsii*-induced increases in steady-state levels of EC IκBα mRNA, as determined by RT-PCR analysis. Increased IκBα mRNA is a sensitive indicator of NF-κB activation since four NF-κB sites are present in its promoter region (17, 35).

NF-κB inhibition by PDTC and TPCK was used to determine if activation of this transcription factor participated in *R. rickettsii*-induced expression of TF mRNA and activity. PDTC and TPCK treatment during infection resulted in abrogation of *R. rickettsii*-induced expression of TF mRNA, as measured by RT-PCR analysis (Fig. 6A). A two-stage clotting assay was used to measure levels of TF procoagulant activity present in uninfected and infected cell lysates. While *R. rickettsii* infection alone resulted in a nearly 10-fold increase in TF activity, such expression was nearly completely inhibited by PDTC or TPCK (Fig. 6B).

DISCUSSION

Superimposed on the necrotic cell injury that occurs during infection of EC with *R. rickettsii* (28), the EC actively responds to infection by altering production of several protein factors which may contribute to the vascular changes that occur during disease. There exists little information as to the mechanism(s) by which the presence of this intracellular pathogen elicits such a response of the host cell or in fact as to whether transcriptional activation of the host cell is a component of this response. The present study provides the first direct evidence for transcriptional activation of a host cell gene during infection and implicates involvement of activation of the transcription factor NF-κB in this transcriptional response.

Both transcriptional and posttranscriptional control mechanisms have been shown to influence steady-state levels of TF mRNA in response to soluble stimuli; however, the relative contribution of each of these control mechanisms is difficult to discern. An increased rate of transcription of the TF gene occurs in EC in response to phorbol myristate acetate (8, 26), tumor necrosis factor (26), and oxidized low-density lipoprotein (10). In contrast, LPS influences posttranscriptional control mechanisms, resulting in enhanced stability that likely derives from the presence of an AU-rich region in the 3′ untranslated region of TF mRNA, which allows for modulation of mRNA stability by inhibitors of transcription and translation (1). It has been reported that LPSs exert no influence on transcription rate (26), but the half-life of TF mRNA has been shown to vary from 2 h during the rapid rise in TF mRNA levels to only 10 min at times when levels decline (8). Our nuclear run-on experiments (Fig. 1) demonstrate that *R. rickettsii* infection induces an increase in transcription rate of TF mRNA at 4 h of infection, coinciding with the occurrence of peak steady-state levels of TF mRNA. However, at 3 h of infection, a time point at which steady-state levels of TF mRNA were increasing, the half-life of TF mRNA was approximately 1.6 h (Fig. 2), which is similar to that noted after EC stimulation with tumor necrosis factor or phorbol ester (1). Since TF mRNA is not present in uninfected EC, we were unable to determine whether the value of 1.6 h during infection represented a significant change over baseline.

The eukaryotic protein synthesis inhibitor CHX was used to determine if de novo host cell protein synthesis was required for induction of TF mRNA during *R. rickettsii* infection. Although CHX alone resulted in increases in steady-state TF mRNA levels, the combination with *R. rickettsii* resulted in superinduction (Fig. 3). This result indicated that host cell protein synthesis is not required in the signal transduction pathways involved in TF expression during infection and that TF behaves as an early-response gene to infection. *R. rickettsii*-induced TF expression, therefore, results largely from direct activation of host cell signal transduction pathways induced by the *R. rickettsii* organism rather than by an autocrine feedback system involving a newly synthesized protein intermediate. This conclusion supports our previous observation of only partial inhibition of TF expression upon inhibition of autocrine cell stimulation by IL-1 (33).

It was recently reported that activation of members of the NF-κB/Rel family of transcription factors occurs during *R. rickettsii* infection of EC (34), and it is postulated that such activation may participate in changes in gene expression during infection. TF expression induced by a variety of soluble mediators is dependent on activation of members of this transcription factor family, and TF expression can be inhibited by using antioxidants and proteasome inhibitors to block NF-κB acti-

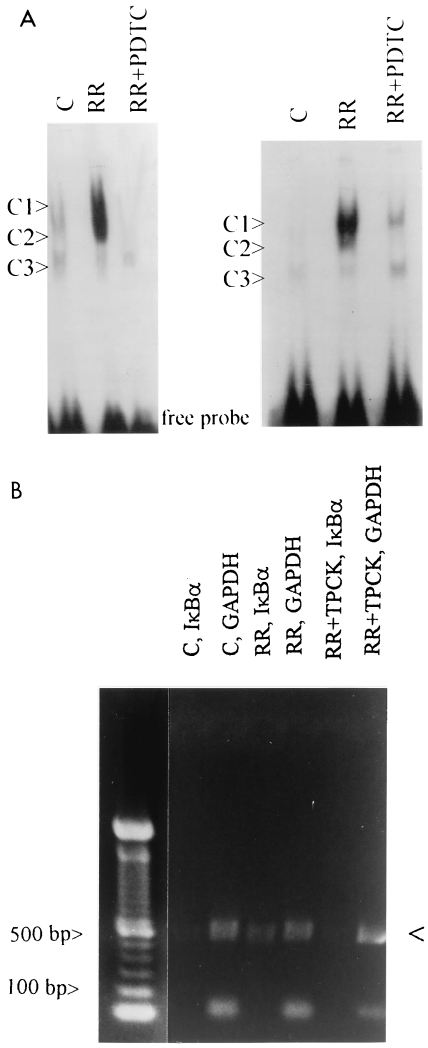

FIG. 5. Effect of PDTC and TPCK on *R. rickettsii*-induced activation of the transcription factor, NF-κB. (A) Activated NF-κB (C1 and C2) was assayed by gel shift assay of nuclear extracts prepared from uninfected EC (C), *R. rickettsii*-infected EC (RR), and infected EC in the presence of PDTC (RR+PDTC) or TPCK (RR+TPCK). C3 represents a nonspecific complex (24, 34). (B) Steady-state levels of IκBα mRNA (566-bp product) and of the housekeeping mRNA species GAPDH (588-bp product) were assayed by RT-PCR in total RNA samples prepared from uninfected EC (C), infected EC (RR), and infected EC in the presence of TPCK (RR+TPCK). Shown are amplification products generated following 30 amplification cycles.

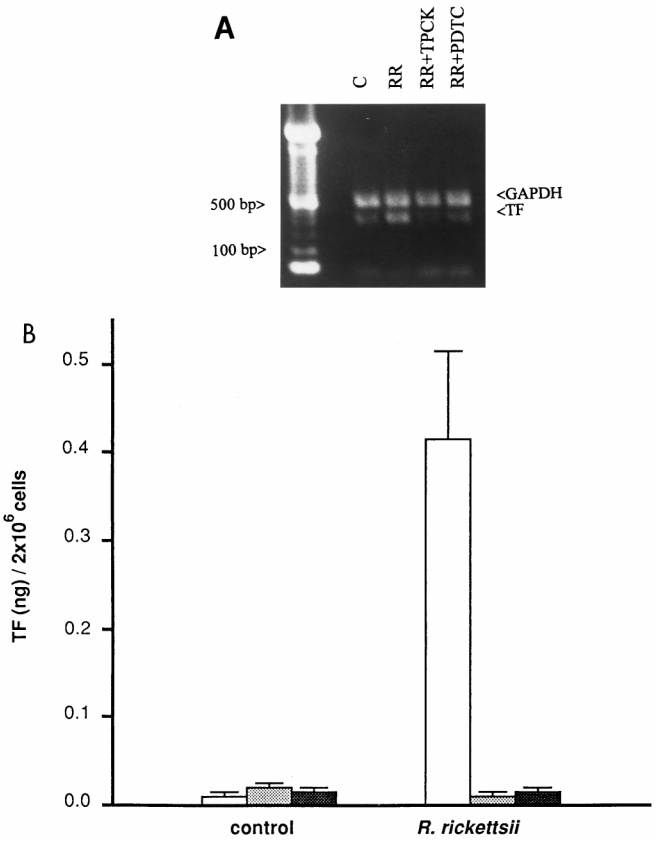

FIG. 6. Effects of PDTC and TPCK on *R. rickettsii*-induced expression of TF mRNA and TF activity. (A) Levels of TF mRNA (398-bp product) and GAPDH mRNA (588-bp product) were analyzed in total RNA samples prepared from uninfected EC (C), *R. rickettsii*-infected EC, (RR), and infected EC in the presence of PDTC (RR+PDTC) or TPCK (RR+TPCK) by RT-PCR analysis using specific primer pairs. Shown are amplification products generated following 30 amplification cycles. (B) TF activity was measured in EC lysates prepared from uninfected EC (control) and from infected EC (*R. rickettsii*) alone (open bars) or in the presence of PDTC (25 μM) (lightly shaded bars) or TPCK (50 μM) (heavily shaded bars) by a two-stage clotting assay. Mean and standard error of the mean are indicated; results shown were obtained from three to nine experiments.

vation (20–22). PDTC, an antioxidant, functions to inhibit an obligatory step in the NF-κB activation pathway requiring a reactive oxygen species (4). TPCK, an inhibitor of a chymotryptic activity associated with the proteasome, blocks activation of NF-κB by inhibiting proteasome-dependent degradation of inhibitory peptides (17). These chemically unrelated compounds were both effective at blocking *R. rickettsii*-induced activation of NF-κB (Fig. 5). Consequently, they blocked *R. rickettsii*-induced increases in TF mRNA and activity (Fig. 6), thus implicating NF-κB activation in *R. rickettsii*-induced expression of TF. This demonstration of involvement of NF-κB is consistent with the absence of a requirement for de novo host cell protein synthesis (Fig. 3), since the NF-κB–IκB complex exists in a preformed yet inactive pool within the host cell cytoplasm (17), and thus its activation can occur even in the presence of protein synthesis inhibitors.

Results of these studies both provide evidence for transcriptional activation of the EC TF gene during *R. rickettsii* infection and demonstrate a role for the transcription factor NF-κB. Furthermore, that *R. rickettsii*-induced increases in TF mRNA occur independently of de novo host cell protein synthesis identifies TF as an early-response gene to this novel intracellular stimulus. Little is known about intracellular signals generated during intracellular *R. rickettsii* infection that may give rise to such a cellular response, but these results suggest that they likely mimic those induced during physiologic stimulation such as by cytokines and growth factors. Further studies of signal transduction and transcription factor activation during infection will provide valuable insight into the mechanisms controlling altered host cell gene expression during *R. rickettsii* infection.

ACKNOWLEDGMENTS

We thank Laura Triou, Laura Hansen, Sarah Lawrence, Terrence Bissoondial, Li Hua Rong, Lisa Domotor, and June Hong for excellent technical assistance and Carol Weed for help in preparation of the manuscript.

This work was supported in part by grants 30616 and 50615 from the

National Heart, Lung, and Blood Institute (NHLBI), grants 17416 and 40689 from the National Institute of Allergy and Infectious Diseases, and NHLBI training grant 07152 (R.-J. Shi).

REFERENCES

1. **Ahern, S. M., T. Miyata, and J. E. Sadler.** 1993. Regulation of human tissue factor expression by mRNA turnover. J. Biol. Chem. **268:**2154–2159.
2. **Bach, R.** 1988. Initiation of coagulation by tissue factor. Crit. Rev. Biochem. **23:**339–368.
3. **Bach, R., R. Gentry, and Y. Nemerson.** 1986. Factor VII binding to tissue factor in reconstituted phospholipid vesicles: induction of cooperativity by phosphatidyl serine. Biochemistry **25:**4007–4020.
4. **Beg, A. A., T. S. Finco, P. V. Nantermet, and A. S. Baldwin, Jr.** 1993. Tumor necrosis factor and interleukin-1 lead to phosphorylation and loss of IκBα: a mechanism for NF-κB activation. Mol. Cell. Biol. **13:**3301–3310.
5. **Bevilacqua, M. P., J. S. Pober, G. R. Majeau, R. S. Cotran, and M. A. Gimbrone.** 1984. Interleukin 1 (IL-1) induces biosynthesis and cell surface expression of procoagulant activity in human vascular endothelial cells. J. Exp. Med. **160:**618–623.
6. **Bevilacqua, M. P., J. S. Pober, G. R. Majeau, W. Fiers, R. S. Cotran, and M. A. Gimbrone.** 1986. Recombinant tumor necrosis factor induced procoagulant activity in cultured human vascular endothelium: characterization and comparison with the actions of interleukin. Proc. Natl. Acad. Sci. USA **83:**4533–4537.
7. **Colucci, J., G. Balconi, R. Lorenzet, A. Pietra, D. Locati, M. B. Donati, and N. Semararo.** 1983. Cultured human endothelial cells generate tissue factor in response to endotoxin. J. Clin. Invest. **71:**1893–1896.
8. **Crossman, D. C., D. P. Carr, E. G. D. Tuddenham, J. D. Pearson, and J. H. McVey.** 1990. The regulation of tissue factor mRNA in human endothelial cells in response to endotoxin or phorbol ester. J. Biol. Chem. **265:**9782–9787.
9. **Drancourt, M., M.-C. Alessi, P.-Y. Levy, I. Juhan-Vague, and D. Raoult.** 1990. Secretion of tissue-type plasminogen activator and plasminogen activator inhibitor by *Rickettsia conorii*- and *Rickettsia rickettsii*-infected cultured endothelial cells. Infect. Immun. **58:**2459–2463.
10. **Fei, H., J. A. Berliner, F. Parhami, and T. A. Drake.** 1993. Regulation of endothelial cell tissue factor expression by minimally oxidized LDL and lipopolysaccharide. Arterioscler. Thromb. **13:**1711–1717.
11. **Gimbrone, M. A., Jr., R. S. Cotran, and J. Folkman.** 1974. Human vascular endothelial cells in culture. Growth and DNA synthesis. J. Cell Biol. **60:**673–680.
12. **Haidaris, P. J., T. W. Wright, F. Gigliotti, and C. G. Haidaris.** 1992. Expression and characterization of a cDNA clone encoding an immunodominant surface glycoprotein of *Pneumocystis carinii*. J. Infect. Dis. **166:**1113–1123.
13. **Hanss, M., and D. Collen.** 1987. Secretion of tissue-type plasminogen activator and plasminogen activator inhibitor by cultured human endothelial cells: modulation by thrombin, endotoxin, and histamine. J. Lab. Clin. Med. **109:**97–104.
14. **Lissitsky, J.-M. Durand, J. Soubeyrand, C. A. Dinarello, and P. Bongrand.** 1995. IL-6 and IL-8 production from cultured human endothelial cells stimulated by infection with *Rickettsia conorii* via a cell-associated IL-1α-dependent pathway. J. Clin. Invest. **96:**2839–2844.
15. **Loskutoff, D. J., M. Sawdey, and J. Mimuro.** 1989. Type 1 plasminogen activator inhibitor, p. 87–115. *In* B. Coller (ed.), Progress in hemostasis and thrombosis, vol. 9. W. B. Saunders, Philadelphia, Pa.
16. **Lyberg, T., K. S. Galdal, S. A. Evensen, and H. Prydz.** 1983. Cellular cooperation in endothelial cell thromboplastin synthesis. Br. J. Haematol. **53:**85–90.
17. **Miyamoto, S., and I. M. Verma.** 1995. REL/NF-κB/IκB story. Adv. Cancer Res. **66:**255–292.
18. **Nawroth, P., and D. Stern.** 1986. Modulation of endothelial cells hemostatic properties by tumor necrosis factor. J. Exp. Med. **163:**740.
19. **Nemerson, Y.** 1988. Tissue factor and hemostasis. Blood **71:**1–8.
20. **Orthner, C. L., G. M. Rodgers, and L. A. Fitzgerald.** 1995. Pyrrolidine dithiocarbamate abrogates tissue factor (TF) expression by endothelial cells: evidence implicating nuclear factor-κB in TF induction by diverse agonists. Blood **86:**436–443.
21. **Parry, G. C. N., and N. Mackman.** 1994. A set of inducible genes expressed by activated human monocytic and endothelial cells contain κB-like sites that specifically bind c-Rel-p65 heterodimers. J. Biol. Chem. **269:**20823–20825.
22. **Parry, G. C. N., and N. Mackman.** 1995. Transcriptional regulation of tissue factor expression in human endothelial cells. Arterioscler. Thromb. Vasc. Biol. **15:**612–621.
23. **Pitlick, F. A., and Y. Nemerson.** 1976. Purification and characterization of tissue factor apoprotein. Methods Enzymol. **45:**37–42.
24. **Read, M. A., M. Z. Whitley, A. J. Williams, and T. Collins.** 1994. NF-κB and IκBα: an inducible regulatory system in endothelial activation. J. Exp. Med. **179:**503–512.
25. **Ruf, W., and T. S. Edgington.** 1994. Structural biology of tissue factor, the initiator of thrombogenesis in vivo. FASEB J. **8:**385–390.
26. **Schleef, R. R., M. P. Bevilacqua, M. Sawdey, M. A. Gimbrone, Jr., and D. J. Loskutoff.** 1988. Cytokine activation of vascular endothelium. J. Biol. Chem. **263:**5797–5803.
27. **Shi, R.-J., P. J. Simpson-Haidaris, V. J. Marder, and D. J. Silverman.** 1996. Increased expression of plasminogen activator inhibitor-1 in *R. rickettsii*-infected endothelial cells. Thromb. Haemostasis **75:**600–606.
28. **Silverman, D. J.** 1986. Infection and injury of human endothelial cells by *Rickettsia rickettsii*. Ann. Inst. Pasteur/Microbiol. **137A:**336–341.
29. **Silverman, D. J., and L. A. Santucci.** 1988. Potential for free radical-induced lipid peroxidation as a cause of endothelial cell injury in Rocky Mountain spotted fever. Infect. Immun. **56:**3110–3115.
30. **Simpson, P. J.** 1987. An improved method for mRNA isolation and characterization of *in vitro* translation products by Western blotting. Gene **56:**161–171.
31. **Sporn, L. A., S. A. Lawrence, D. J. Silverman, and V. J. Marder.** 1993. E-selectin-dependent neutrophil adhesion to *Rickettsia rickettsii*-infected endothelial cells. Blood **81:**2406–2412.
32. **Sporn, L. A., P. J. Haidaris, R.-J. Shi, Y. Nemerson, D. J. Silverman, and V. J. Marder.** 1994. *Rickettsia rickettsii* infection of cultured human endothelial cells induces tissue factor expression. Blood **83:**1527–1534.
33. **Sporn, L. A., and V. J. Marder.** 1996. Interleukin-1α production during *Rickettsia rickettsii* infection of cultured endothelial cells: potential role in autocrine cell stimulation. Infect. Immun. **64:**1609–1613.
34. **Sporn, L. A., S. K. Sahni, N. B. Lerner, V. J. Marder, D. J. Silverman, L. C. Turpin, and A. L. Schwab.** 1997. *Rickettsia rickettsii* infection of cultured human endothelial cells induces NF-κB activation. Infect. Immun. **65:**2786–2791.
35. **Sun, S.-C., P. A. Ganchi, D. W. Ballard, and W. C. Greene.** 1993. NF-κB controls expression of inhibitor IκBα: evidence for an inducible autoregulatory pathway. Science **259:**1912–1915.
36. **Teysseire, N., D. Arnoux, F. George, J. Sampol, and D. Raoult.** 1992. von Willebrand factor release, thrombomodulin activity and tissue factor expression in *Rickettsia conorii*-infected endothelial cells. Infect. Immun. **60:**4388–4393.
37. **van den Berg, E. A., E. D. Sprengers, M. Jaye, W. Burgess, T. Maciag, and V. W. M. van Hinsbergh.** 1988. Regulation of plasminogen activator inhibitor-1 mRNA in human endothelial cells. Thromb. Haemostasis **60:**63–67.
38. **Wagner, D. D., J. B. Olmsted, and V. J. Marder.** 1982. Immunolocalization of von Willebrand protein in Weibel-Palade bodies of human endothelial cells. J. Cell Biol. **95:**355–360.
39. **Woodward, W. E., and R. B. Hornick.** 1990. Infectious diseases and their etiologic agents, p. 1082–1100. *In* G. L. Mandell, R. G. Doublas, Jr., and J. E. Bennett (ed.), Principles and practice of infectious diseases. Churchill Livingston, New York, N.Y.

Editor: R. E. McCallum